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Microbiol, Immunol (1985) Vol 29 p. 959-972.
Lymphokines (N.Y.) (1984) vol. 9 p. 127-52.
J. of Immunology (U.S.) (1985) Vol. 134 p. 390 1-6
Molecular Immunology (1984) Vol. 21 No 12 p. 1229-36
Microbiology and Immunology (1986) Vol. 30 No. 4 p.
373-388.

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C6F
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Selected US specifications from IPC sub-classes A61K
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(54) Monoclonal antibodies recognizing human interleukin-2-receptor

(57) Hybrid cell lines have been produced for the production of monoclonal antibody to an antigen found on activated human lymphocytes, the interleukin-2-receptor (IL-2-R) to the antibody so produced, and to therapeutic and diagnostic methods and compositions employing this antibody.

The antibody may be in the form of a chimeric animal-human antibody recognising IL-2-R wherein the F_c region is obtained from a human and the F_{ab} region is obtained from an animal.

GB 2 188 941 A

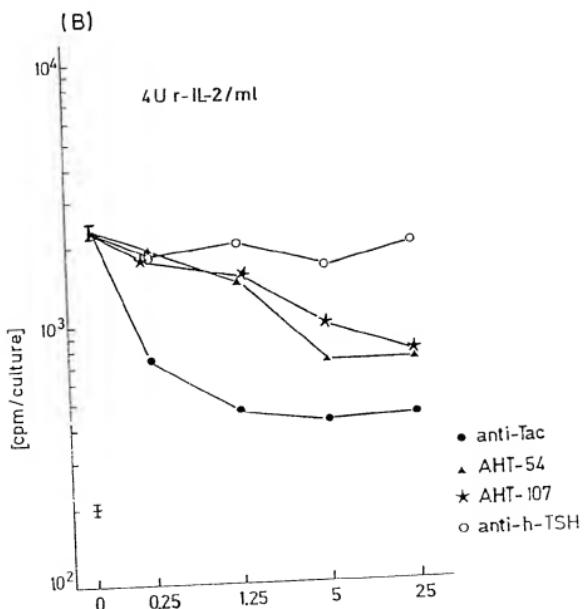


FIG. 1a

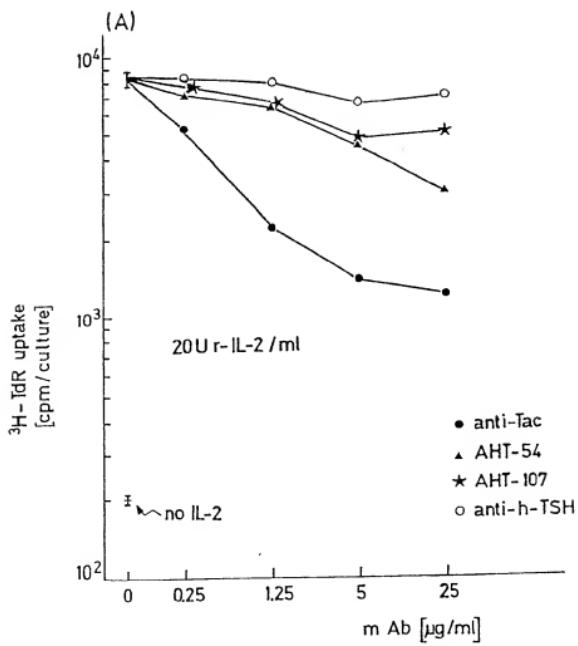


FIG. 1b

FIG. 2

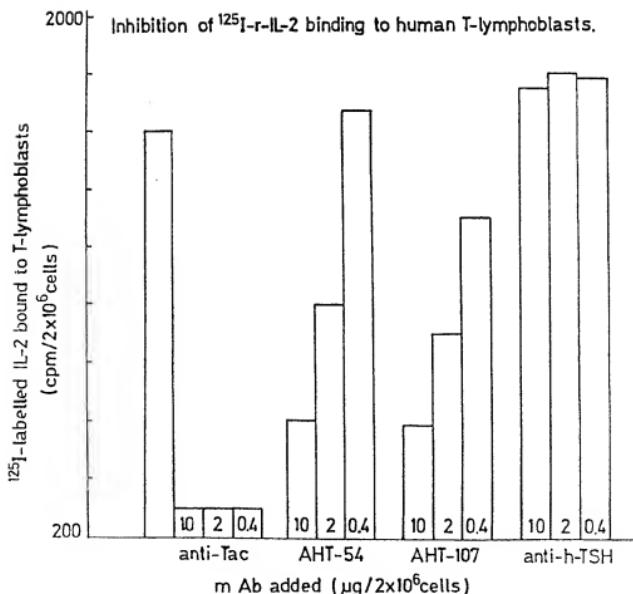
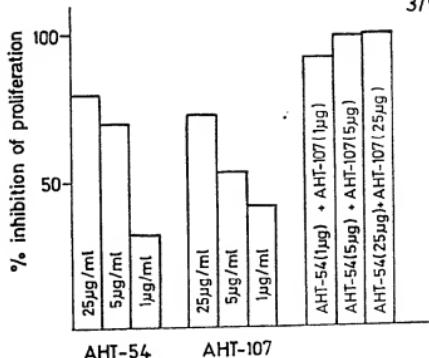


FIG. 4

09058 - 14 APR. 86

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4/9

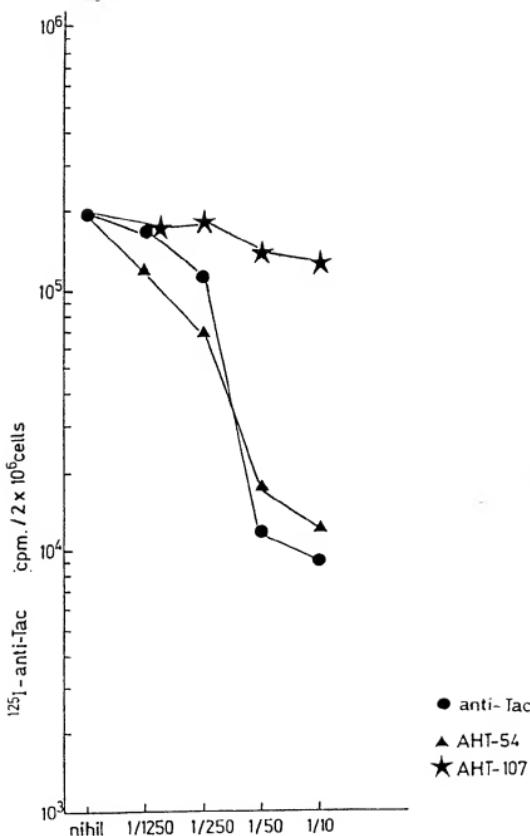


FIG. 3a

L9058 - 14 APR. 86

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5/9

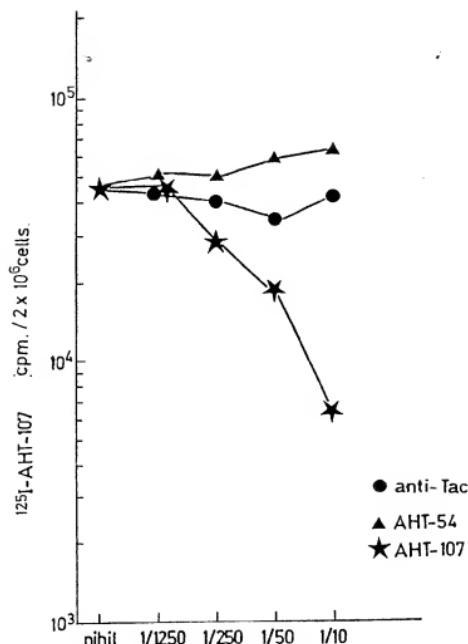


FIG. 3 b

L9058 - 14 APR. 86

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6/9

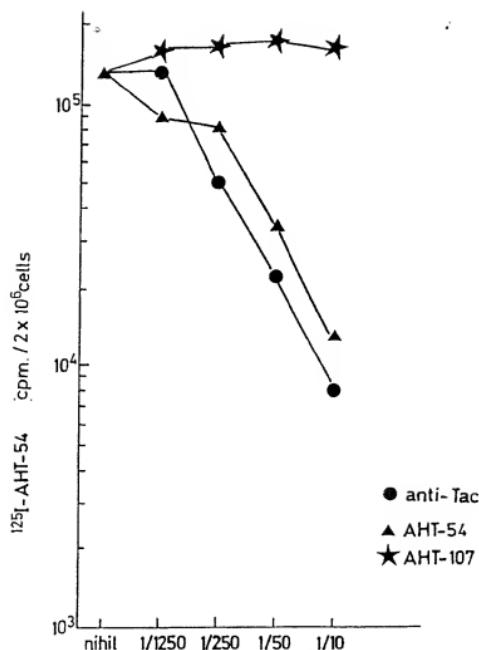


FIG. 3c

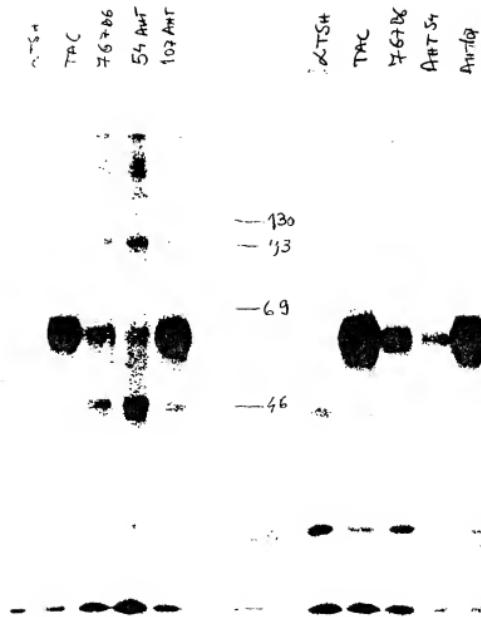


FIG. 5

8/9

ONE PARAMETER ANALYSIS

512

10000

MEAN
22.36
SD
27.62
CV
123.52
LCV
0
RCV
678.27
HCV
67.20

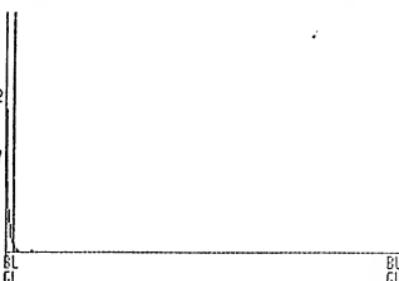


FIG. 6

ONE PARAMETER ANALYSIS

512

10000

MEAN
68.26
SD
33.88
CV
49.52
LCV
49.18
RCV
53.20
HCV
40.51

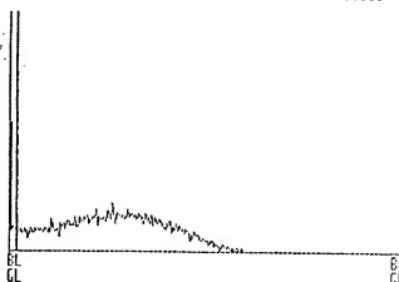


FIG. 7

ONE PARAMETER ANALYSIS

512

10000

MEAN
67.19
SD
34.57
CV
51.45
LCV
50.10
RCV
58.26
HCV
49.07

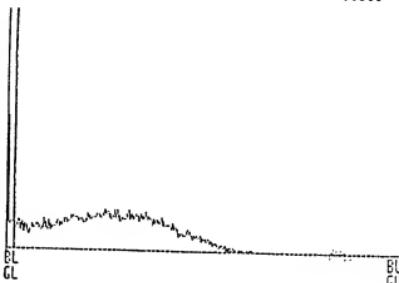


FIG. 8

09058 - 14 APR. 86

2188941

FIG. 9

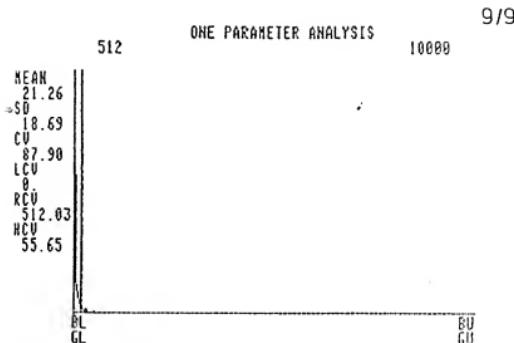


FIG. 10

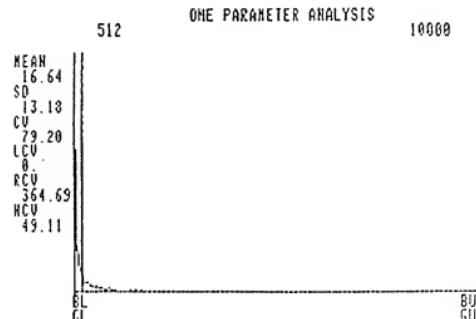
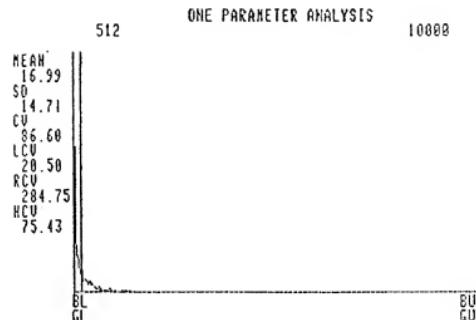


FIG. 11



SPECIFICATION

Monoclonal antibodies recognizing human interleukin-2-receptor

5 This invention relates generally to new hybrid cell lines and more specifically to hybrid cell lines for production of monoclonal antibody to an antigen found on activated human lymphocytes, the interleukin-2-receptor, to the antibody so produced, and to therapeutic and diagnostic methods and compositions employing this antibody.

10 15 INTRODUCTION

For many, if not all cells, the initial trigger for proliferation appears to be the interaction of growth factors with the cell surface growth factor receptor. Activation of the growth factor receptor leads in turn to yet undefined cytoplasmic signalling systems.

Resting T lymphocytes are long living cells in the G₀ phase of the cell cycle. They only enter proliferative cycles under antigenic stimulation in the presence of a T cell growth factor, interleukin 2 (IL-2). Receptors for IL-2 are not detectable on the surface of resting T cells. Expression of IL-2 receptors (IL-2R) is the consequence of interaction of antigen presenting cells with the antigen receptor. As shown recently, IL-2 receptor expression is a transient even and repeated restimulation by lectins (Cantrell, P.A., and K.A. Smith, (1984). Science (Wash. DC) 224:1312); (Osawa, H., and Diamantstein, T. (1984). J. Immunol. 132:2445) or the antigen (Reske-Kunz, A.B., D.v. Steldern, E. Rüde, H. Osawa and T. Diamantstein, (1984). J. Immunol. 133:1356) is required for continuous IL-2 receptor expression and consequently for long term cell growth.

Since, IL-2-R are expressed exclusively on activated lymphocytes monoclonal antibody (mAb) that reacts with the IL-2-R may be useful as specific and selective immunosuppressive agent. Furthermore, such antibodies may serve as diagnostic reagents in order to detect qualitatively and quantitatively activated lymphocytes as well as neoplastic cells expressing IL-2-R.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a composition of at least two monoclonal antibodies recognizing human interleukin-2-receptor capable to inhibit interleukin 2 induced lymphocyte proliferation. In an preferred embodiment one or more of the antibodies are of the IgG₁ class. In another preferred embodiment the antibody composition is capable to inhibit interleukin-2 binding to the receptor. The present invention provides monoclonal antibodies of the IgG₁ class recognizing human interleukin-2-receptor capable to inhibit interleukin-2 binding to the receptor and in particular

antibodies capable to inhibit interleukin-2 dependent lymphocyte proliferation. The antibodies of the present invention can be used for preparation of chimeric animal-human anti-

70 bodies recognizing human interleukin-2-receptor wherein the constant Fc region of the immunoglobulin is obtained from human and the variance Fab region is obtained from an animal. Preferably the Fab region is obtained

75 from mice.

The present invention further provides hybridoma cell lines, characterized by the production of monoclonal antibodies of the IgG₁ class recognizing human interleukin-2-receptor.

80 Particular preferred are the hybridoma cell lines having the NTCC designation number . . . and . . .

Human T-lymphoblasts expressing IL-2-R were prepared by known methods (Osawa, H.,

85 and Diamantstein, T. (1983) J. Immunol. 130:51.) were used to produce a mouse monoclonal antibody against IL-2-R by the technique of Köhler and Milstein (Köhler, G., and C. Milstein, (1975) Nature 256:495). The fusion resulted in two hybrid clones AHT-54 and AHT-107 producing anti-IL-2-R antibodies of IgG₁ subclass. The hybrid clones secreting anti-IL-2-R antibodies were selected as preferred embodiment of the present invention.

95 Both mAb i) inhibit binding of 125I labelled IL-2 to IL-2-R positive human lymphocytes, ii) inhibit IL-2 dependent proliferation in vitro and iii) precipitates the identical cell surface molecule of 55KD, the IL-2 binding protein. Competitive binding of AHT-54 and AHT-107 revealed that they recognize different epitopes of the IL-2-R molecule.

AHT-107 is different from anti-Tac (Uchiyama, T. et al., (1981) J. Immunol. 126:1398) because competitive inhibition studies revealed that they recognize two different epitopes of the IL-2-R molecule; AHT-107 is also different from 7G7 B6, a recently published

100 antihuman IL-2-R mAb (A. Rubin, C. Kurman, E. Biddison, D. Goldman, and L. Nelson (1985) Hybridoma Vol. 4:91), because in contrast to 7G7 B6 AHT-107 inhibits binding of IL-2 to the IL-2-R as well as IL-2 dependent proliferation of lymphocytes.

115 Both mAb react specifically with activated lymphocyte (T and B) but not with resting lymphocytes or other non-lymphoid cells. This statement is based on FACS-analysis data (Fig. 6-11).

120 According to previous studies in animal models such Ab reacting with the rat (ART-18) and with the mouse IL-2-R (AMT-13 and M7/20) has been shown to inhibit selectively and specifically i) local GVH-reaction (Diamantstein, T. and H. Osawa, (1986), Immune Rev. 92 in press.) ii) cardiac allograft rejection (L. Kirkman, E. Kelley, A. Kolton, J. Schoen, A. Ythier and B. Strom, (1985), Transplantation 40:719), (L. Kirkman, L.V. Barrett, N.

125 Gaulton, E. Kelley, A. Ythier and B. Strom,

130

(1985), *J. Exp. Med.* 162: 358.) and iii) T-cell-mediated autoimmune reaction such as acute autoimmune encephalomyelitis and adjuvant arthritis induced by T-cell transfer (Wekerle, H. and T. Diamantstein, (1986), *Autoimmunity: Experimental and Clinical Aspects* Eds: R.S. Schwarz, N.R. Rose, Ann. New York Acad. Sci., In press.)

The anti-IL-R monoclonal antibodies of the present invention are also useful as therapeutic agents in clinical syndromes which are associated with pathological proliferation of IL-2 dependent cells. Thus, for example, hyperimmune syndromes such as Host versus Graft(HVG) Graft versus Host (GvH) diseases and autoimmune diseases (e.g. multiple sclerosis, autoimmune diabetes, Crohn's disease) may be treated. In a preferred embodiment of the present invention, the anti-IL-2-R monoclonal antibodies are used as therapeutic agents directly without further modification thereof. Furthermore, the invention includes preparation of anti IL-2-R chimeric antibodies using human heavy chain of different classes and subclasses in combination with the variable region of the AHT-54 and AHT-107 mAb, in order to optimise for therapeutic use.

Alternatively, the antibodies may be coupled to drugs including cytotoxic agents. The monoclonal antibodies of the present invention are capable of recognizing specifically cells expressing IL-2 receptors, inhibiting their function and of eliminating them selectively.

The monoclonal antibodies of the present invention are also useful diagnostic reagents for cells which contain IL-2-R either on the cell surface or within the cells and in body fluids. Thus by means of the present invention, cells containing IL-2-R may be identified in samples having different kinds of cells. Localization of IL-2-containing cells is possible in cultured cell colonies or in tissue specimens. When used in this manner the monoclonal antibodies are preferably coupled to fluorescent, color-forming substances such as an enzyme or chromophor, or a radioactive substance (ELISA, RIA).

DETAILED DESCRIPTION OF THE INVENTION

The following description is intended to illustrate this invention without limiting the same in any manner especially with respect to substantially functional equivalents of hybridomas and monoclonal antibodies as described herein.

55 I. Production of mAb Source of IL-2-R

IL-2-R expressing cells were prepared as described using human-T-lymphoblast. Mixed human peripheral blood lymphocytes were stimulated with 3 mg/ml of concanavalin A (Con A) for 3 days. The cells were converted, treated with α -methyl mannoside (20 mg per ml), washed and used as immunogens in culture

RPMI medium (Seromed GmbH, München, F.R.G.) supplemented with 2×10^{-3} M L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, and 70 5 to 10% (v/v) fetal calf serum (FCS; batch No. 104; Seromed GmbH).

Immunization, cell fusion, cloning, and production of monoclonal antibodies (mAb)

75 Ten-week-old BALB/c mice were primed with 2×10^7 T lymphoblasts. The cells were injected in 0.1 ml portions (10^6 cells) subcutaneously into the footpads and into the necks of the mice as well as i.v. (10^7 cells in 0.5 ml). Four weeks later, the mice were challenged i.v. with 10^7 T-lymphoblasts. Three days later, spleen cells from the immunized mice were fused with X63-Ag8.653 mouse myeloma cells in the presence of polyethylene glycol (Köhler and Milstein, (1975), *Nature* 256:495, as modified by Lemke H., G.J. Hämerling, C. Höhmann and K. Rajewsky, (1978), *Nature* 271:249). Fused cells suspended in HAT medium were distributed into each well of ten 24-well tissue culture plates (1 to 2×10^6 spleen cells/well). Supernatants of the wells in which vigorous growth was observed after 3 to 4 wk were screened for their capacity to bind a) human T lymphoblasts, b) 95 mouse T lymphoblasts, and c) human thymocytes attached to the surface of the wells of microtiter plates. Cell-bound immunoglobulin was then detected by enzyme-linked immunosorbent assay (ELISA) as described (Kincade, 100 P.W., G. Lee, L. Sun, and T. Watanabe, (1981), *J. Immunol. Methods* 42:17.) by using β -galactosidase-coupled sheep IgG_{2b}² anti-mouse immunoglobulin (New England Nuclear, Drieieich, F.R.G.) as a second antibody. The 105 hybridomas grown in HAT or RPMI medium that constantly produced antibodies binding specifically to human T lymphoblasts were selected. Supernatants of growing hybridomas were repeatedly tested and selected for hybridomas producing supernatants active in the functional assay (inhibition of the T-lymphoblast response to IL-2) as well as the absorption assay (inhibition of the capacity of T lymphoblasts to absorb IL-2 after preincubation). 110 Positive hybridomas were cloned by limiting dilution with mouse thymocytes used as a feeder layer. The clones were retested and expanded. The supernatants of the relevant clones were used for isolation and purification 120 of the mAb.

Purification of the mAb

As tested in the Ouchterlong double immunodiffusion test with rabbit anti-mouse IgM, 125 IgA, IgG1, IgG2a, IgG2b, and IgG₃ sera (Miles Laboratories, Ltd., Slough, England), the hybridoma clone AHT-54 and AHT-107 were found to produce IgG1 antibodies. Excepting the initial screening experiments, in which un-

following experiments were performed with purified IgG1. Purification was achieved by successive binding/elution from protein A-Sepharose (Pharmacia Fine Chemicals) according to the method described by Ey et al., (Ey, P.L., S.J. Prowse and C.R. Jenkin, (1978), Immunochimistry 15:429). About 600 ml of the culture supernatants, brought to pH 8.0, were passed over a 5 ml protein A-Sepharose column equilibrated in 0.1 M sodium phosphate buffer (pH 8.0) IgG1 was eluted from the column with 0.1 M sodium citrate buffer (pH 6.0). The purified antibody was then dialyzed against a buffer containing 0.01 M HEPES (pH 7.4) and 0.9% NaCl. The purity of mAb was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis performed in reducing conditions as described (Laemmli, U.K. 1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature 227:429). The protein concentration of the purified IgG1 was determined by absorption of ultraviolet light at 280 nm, assuming an extinction coefficient (1% w/v:1cm) of 14, and by the method of Lowry et al. 1951 (Lowry, O.H., N.J. Rosebrogh, A.L. Farr, and R.J. Randall (1951). J. Biol. Chem. 193:265.) with bovine serum albumin (BSA) used as the standard.

Recombinant Interleukin-2 provided from Sanofi Mena was used. ¹²⁵I-labelled recombinant IL-2 was produced from NEN.

Labelling of the mAB with ¹²⁵J

mAb were labelled with ¹²⁵J according to McConahey and Dixon (McConahey, P.J., and F.J. Dixon, (1980) Methods Enzymol, 70:210). Briefly, 20 µg of IgG1 dissolved in 60 µl of Na¹²⁵J(100 Ci ml⁻¹, carrier-free; Amersham 40 Buchler). Ten microliters of chloramine-T (2.5 mg ml⁻¹ in 0.05 M Na-P) were added to the mixture. After 45 sec of incubation at room temperature, 20 µl of Na₂S₂O₈ (3 mg ml⁻¹ in 0.05 M Na-P) were added to the tube. The mixture was immediately loaded onto a 1.0 ml Sephadex G-75 column (prewashed with 0.05 M Na-P containing 4% BSA and washed consecutively with 0.05 M Na-P until the eluate was protein-free), and the radiolabel in the excluded fraction was collected.

Detailed description of the drawings:

Figure 1

Inhibition of IL-2 dependent human T-lymphoblast proliferation by different mAbs.

2×10^6 human T-lymphoblasts were incubated for 3 days in 0.2 ml of medium containing the indicated amounts of recombinant IL-2 (Fig. 1a, 20U/ml; Fib. 1b, 4U/ml) in the absence or presence of the different mAbs, anti-Tac (-○-), HT-54 (-Δ-), AHT-107 (-☆-) and of a control mAb anti-human-TSH (-○-). The cells were pulsed with ³H-thymidine for the last 4 h of the incubated period. Incorporation of ³H-thymidine was measured

according to the standard procedure (Diamantstein et al, Mol. Immunol., (1984), 21:1229.

Figure 2

Synergistic action of AHT-54 and AHT-107 mAbs on IL-2 dependent proliferation

T-lymphoblasts were cultured with 20U/ml of r-IL-2 for 3 days (for detail see Fig. 1) in the presence of either AHT-54 or AHT-107 mAb or in combination of both mAbs.

Figure 3

Competition for the binding of ¹²⁵I-labelled antibodies

2×10^6 human T blasts were first suspended in 100 µl of a binding buffer (PBS=0.5% BSA/10 mM Na₂S₂O₈) containing different dilutions of the mAbs anti-Tac (-○-), AHT-54 (-Δ-) and AHT-107 (-☆-). The suspensions were mixed with 100 µl of a 1:40 dilution of the ¹²⁵I-labelled mAb.

The mixture was incubated for 1h at 4°C.

The relative amount of ¹²⁵I-labelled mAb (cpm) bound to the pelleted cells was measured by using a gamma-radiation counter after washing them twice with the binding buffer.

Figure 4

Inhibition of ¹²⁵I-IL-2 binding to human T blasts by different mAbs

2×10^6 human T blasts were first incubated for 30 min at 37°C in 0.25 ml of a buffer (RPMI/Hepes/BSA/Na₂S₂O₈) containing the indicated amounts of different mAbs. The incubation was further continued at 37°C in the presence of ¹²⁵I-IL-2. After 40 min, the incubation mixture was centrifuged to pellet the cells and the pelleted cells were taken up in 100 µl of the buffer and overlaid on the oil phase consisting of dibutylphthalate/olive oil (10+3). After centrifugation the tips of the tube containing the cell pellets were cut out and counted in a gamma-radiation counter.

Figure 5

SDS-PAGE analysis of immunoprecipitates with different mAbs

2×10^7 human T blasts were surface-iodinated with 0.5 mCi of Na [¹²⁵I] and lysed in 0.5 ml of the lysis buffer. The lysate was centrifuged and preabsorbed with 1/5 volume of protein A-sepharose beads (10 µl) through a bridge antibody rabbit anti-mouse IgG. After 1h at 4°C the beads were washed three times with a buffer containing 50mM Tris-HCL, pH 8.3, 450 mM NaCl, 5mM KI, 0.02% Na₂S₂O₈ and 0.5 Nonidet P-40, and extracted with 100 µl of the sample buffer. 50 µl aliquots of the extracts were subjected to SDS-PAGE analysis either under non-reducing (lanes 1-6) or reducing (lanes 7-12) conditions. The mAbs used were control mouse UPC-10 ascites (lanes 1 & 7), anti Tac ascites (lanes 2 & 8),

AHT-54 ascites lanes 4 & 10), AHT-107 ascites (lanes 5 & 11) and AHT-107 culture supernatants (lanes 6 & 12).

5 *Figures 6 to 11*

FACS-analysis

Human peripheral blood cells (HPBL) and activated T-lymphoblasts derived from HPL were incubated at 4°C in presence of 0.1 % NaN₃ for 30 min with AHT-54 or AHT-107 mAB (1:1000 ascites fluid and as negative control with a TSH, washed and stained using saturating amount of goat anti-mouse IgG labelled with FITC. Fluorescence activated cell sorter analysis performed with Epics V.

Fig. 6 shows the negative control. The α-TSH antibody did not bind to the T-lymphoblasts. In Fig. 7 and 8 it is shown that AHT 54 (Fig. 7) and AHT 107 (Fig. 8) are capable to bind to the lymphoblasts. Fig. 9 to 11 are referring to the same experiment except the lymphoblasts are substituted by HPBL. With none of the three antibodies a reaction occurs.

25 CLAIMS

1. A composition of at least two monoclonal antibodies recognizing human interleukin-2-receptor capable to inhibit interleukin-2 dependent lymphocyte proliferation.
- 30 2. A composition according to claim 1, wherein at least one of the monoclonal antibodies is of the IgG, class.
3. A composition according to any of claims 1 and 2 inhibiting interleukin-2 binding to the receptor.
- 35 4. A monoclonal antibody of the IgG, class recognizing human interleukin-2-receptor capable to inhibit interleukin-2 binding to the receptor.
- 40 5. A monoclonal antibody according to claim 4 capable to inhibit interleukin-2 induced lymphocyte proliferation.
6. A chimeric animal-human antibody recognizing human interleukin-2-receptor.
- 45 7. A chimeric antibody according to claim 6, wherein the F_c region is obtained from human and the Fab region is obtained from an animal.
8. A chimeric antibody according to any of claims 6 and 7, wherein the Fab region is obtained from mice.
- 50 9. Hybridoma cell lines, characterized by the production of monoclonal antibodies of the IgG, class recognizing human interleukin-2-receptor.
- 55 10. The hybridoma cell lines of claim 9 having the NTCC designation number . . . and